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Vasodepressor Effect of Atrial Natriuretic Peptides in the Quail, *Coturnix coturnix japonica*

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ABSTRACT—An intravenous injection of α-human atrial natriuretic peptide-(1-28) (α-hANP) caused dose-dependent decreases in both arterial pressure and heart rate in the urethane-anesthetized quail. The hypotension always preceded the bradycardia. The ED_{50} for the vasodepressor effect (58.2±4.6 pmol/100 g, n=28) was smaller than that for the bradycardial effect (107.2±8.9 pmol/100 g, n=23). The hANP peptides with 3–6 amino acids removed from the NH_2-terminus were as potent as α-hANP, but the peptide with 4 amino acids removed from the COOH-terminus had only about half the vasodepressor potency of the parent molecule. Among the peptides of hANP in which an amino acid within the ring structure was modified, only des-Gly^{9} hANP, [(Met (O)^{12}] hANP, and [Asn^{13}] hANP had decreased the potency. Formation of the ring structure between the 7th and the 23rd position with an ethylene linkage instead of a disulfide bond did not change the potency. These results suggest that the amino acid residues at the COOH-terminus and some amino acids within the ring structure of hANP are important for the expression of its vasodepressor activity in the quail.

INTRODUCTION

Since the discovery of a potent diuretic and natriuretic substance in the rat atrium by de Bold *et al.* [1], a growing number of studies have been performed in mammals to elucidate the structure and biological actions of this substance. These studies have demonstrated that the stored form of the natriuretic factor has 126 amino acids in the rat and human, and is named cardiodilatin-126, γ-atrial natriuretic peptide (ANP) or pro-atrial natriuretic factor [see 2]. Later studies have shown that the circulating form of ANP has 28 amino acids that comprise the COOH-terminus of the propeptide, and that this (1-28) molecule, termed α-ANP by Kangawa and Matsuo [3], is most potent among the endogenous peptides of ANP. In addition to natriuretic and diuretic effects, ANP has been shown to exhibit a potent vasodepressor effect [1, 4, 5] and a cardiosuppressive effect [6–8] in several species of mammals. The mechanism that causes hypotension could involve a decrease in total peripheral resistance, a decrease in cardiac output, and/or a decrease in blood volume via diuresis, but the actual mechanism has not yet been determined [2]. In nonmammalian vertebrates, the effect of ANP on blood pressure is still controversial. It has been shown that synthetic ANP peptides decrease arterial pressure in the chicken [9] and dogfish [10], while they have no effect on arterial pressure in the toadfish [11], and increase it in the rainbow trout [12].

In the present study, we attempted to examine the effects of α-human ANP (α-hANP) on arterial pressure and heart rate in the quail. We also examined structure-activity relationships of hANP peptides for the effect on blood pressure to examine the structural requirement of ANP molecules for quail vascular receptors. The results were compared with other *in vivo* studies on the natriuretic activity in the rat [13, 14] and antidipsogenic activity in the rat [15], and *in vitro* studies on the spasmolytic activity of the rat and rabbit aortic strips [14, 16] and of the chick rectum [13, 14].

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MATERIALS AND METHODS

Animals

Male Japanese quail, *Coturnix coturnix japonica*, were purchased from a local dealer at the age of 4 weeks. They were kept individually in wire cages ($21 \times 12 \times 17$ cm^3) under a short daily photoperiod (8L : 16D) at 25 ± 1°C for more than 2 weeks before use. Quail diet (Nippon Haigo shiryo, Yokohama) containing 150 meq/kg of Na and tap water were freely available until the day of experiment. The birds weighed 106 ± 2 g (n=28) at the time of experiments.

Surgery

The birds were lightly anesthetized by an intramuscular injection of urethane (2 g/kg) in the breast region and fixed on an operating board. It has been reported that urethane anesthesia is suitable for cardiovascular studies because of its little effects on cardiovascular reflexes [17]. After tracheotomy, a polyethylene tube (PE10, Clay Adams) was inserted into the right atrium through the right external jugular vein for injection of ANP, and a cannula assembly was inserted into the right common carotid artery for measurement of arterial pressure (Fig. 1). Care was taken not to damage the vagus nerve and other nerves that innervate the carotid sinus. Almost no bleeding was observed during surgery. The jugular cannula was filled with isotonic saline, and the arterial cannula was filled with isotonic saline which contained 100 units/ml of heparin.

Measurement of arterial pressure and heart rate

The cannula in the carotid artery was connected to a small semiconductor-type pressure transducer (PML-500GC, Kyowa Electric Instruments Co., Ltd.,Tokyo) via a short silicone-rubber tube (Fig. 1). Since the transducer was connected to a carrier amplifier (Type 3126, Yokogawa Electric Works Ltd, Tokyo) by a long, flexible cord, the cannula would not slip out of the artery even when the fixed birds move slightly under anesthesia. The original pressure waves and the integrated waves (the mean pressure) were amplified and recorded with a recorder (Rectigraph-8K, NEC San-ei, Tokyo). The original waves after amplification were also introduced into a tachometer (#1322, NEC San-ei, Tokyo) for measurement of heart rate, and they were stored in a data recorder (R-260, TEAC, Tokyo) for further analyses.

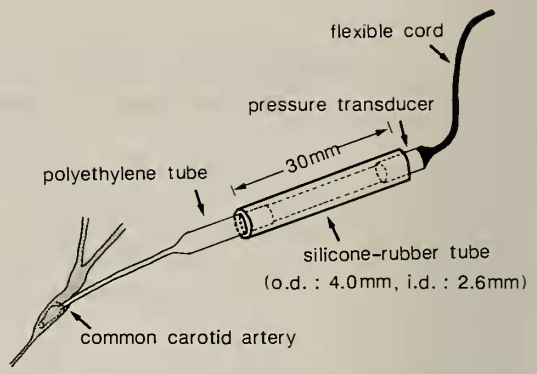

FIG. 1. Cannula assembly for the measurement of arterial pressure from the common carotid artery of the quail. The polyethylene tube (o.d.: 2.7 mm, i.d.: 1.8 mm) is narrowed to 0.6–0.8 mm by pulling the tube after heating, and then its tip is dilated by heating. The polyethylene tube and silicone-rubber tube (SH-3, Create Medic, Yokohama, Japan) are filled with heparinized saline (100 units/ml). The outer diameter of the transducer is 2.8 mm. Since the transducer is not fixed, the cannula does not escape from the artery even though the bird moves to some extent during the measurements. Another advantage of this system is that, because the cannula is rather thick and short, the pulse wave can be recorded with a small distortion.

Experimental protocol

In the first experimental group (n=10), the ED_{50} of α-hANP for its effects on arterial pressure and heart rate was determined. Intravenous injections of 0, 0.05, 0.1, 0.2, 0.5, 1 or 2 μg/100 g body weight of α-hANP in 0.05 ml of 0.9% NaCl were given twice for each dose in random order. Each injection was followed by flushing of the cannula (dead space: 0.01 ml) with 0.03 ml of 0.9% NaCl. The injection was repeated after the arterial pressure returned to the level before injection, since it was shown in preliminary experiments that repeated injections of 0.5 μg of α-hANP given in this way generated reproducible effects.

In the second experimental group (n=18), the structure-activity relationship of the vasodepressor effect was examined using various analogs of

hANP. For this purpose, 0, 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/100 g of α-hANP (hANP-(1-28)) were first injected in that order, then three or four of the analogs were injected at the doses of 0.1, 0.3 and 1 μg/100 g in that order. The analogs of hANP used were [Ile^{12}] hANP-(1-28) (α-rat ANP), [Met(O)^{12}] hANP-(1-28), hANP-(4-28), hANP-(5-28), hANP-(7-28), [Nle^{12}] hANP-(7-28), [D-Ala^{9}] hANP-(7-28), [Asn^{13}] hANP-(7-28), des-Gly^{9} hANP-(7-28), hANP-(5-25), hANP-(7-23), and [Asu^{7,23}] hANP-(7-23). The order of injection of the analogs was random. After the last injection of the analogs, injections of 0.1, 0.3 and 1 μg/100 g of α-hANP were repeated to confirm the reproducibility of results. All hANP-related peptides were synthesized by the liquid-phase method [18] at the Peptide Institute Inc. (Osaka, Japan).

Statistical analyses

Since it was found that decreases in arterial pressure and heart rate were greater when the pre-injection levels were higher, the decreases were expressed in terms of percentages from pre-injection levels. The ED_{50} of α-hANP was calculated from all data of 28 birds used in this study. For calculation of the ED_{50}, changes in arterial pressure or heart rate at doses between 0.05 and 2 μg were fitted to a logistic curve by each bird, and the dose that produced a half-maximal respose was obtained from the curve. The curve fitting was executed by the Newton-Raphson algorithm based on estimates of maximum likelihood [19]. The changes in arterial pressure and heart rate at each dose were compared with the changes observed after injection of saline by the paired t-test. In the experiment to examine the vasodepressor potency of analogs of hANP relative to α-hANP, the potency ratio was calculated from the ratio of the ED_{50} of hANP to that of an analog. In this case, the ED_{50} of α-hANP was calculated with data obtained at doses between 0.1 and 1 μg, as was done for each analog. The response to 2 μg of α-hANP was used only to assess the maximum response. All results are expressed as means±SE of the mean.

RESULTS

Dose-response relationship for α-hANP

The mean, resting arterial pressure of the quail before injection was 84.1±2.3 mmHg (n=28). The arterial pressure decreased immediately after injection of α-hANP, and the decreases become greater as the dose increased (Fig. 2). The largest decrease at high doses was about 50% of the level before injection. The ED_{50} calculated by the logistic-log transformation was 179±14 ng (58.2±4.6 pmol)/100 g body weight (n=28) (Fig. 3). The significant decrease in arterial pressure was obtained at 100 ng/100 g, and 9 out of 28 birds decreased their arterial pressure at 50 ng/100 g (Fig. 2). The decreased pressure continued for longer as the dose increased (Fig. 2).

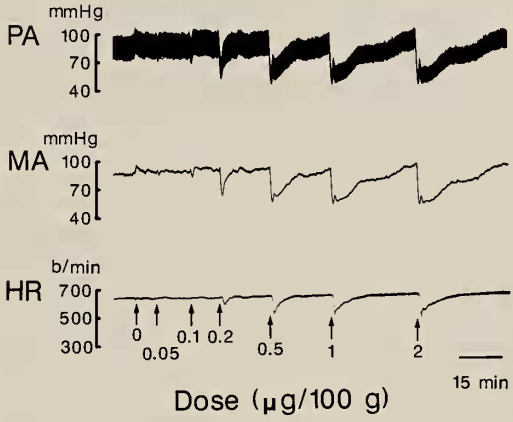

FIG. 2. Changes in arterial pressure (PA), mean PA (MA), and heart rate (HR) after injection of 0–2 μg/100 g body weight of α-hANP into the jugular vein of a quail.

The mean heart rate of the quail before injection was 515±22 beats/min (n=28). The heart rate decreased after injection of α-hANP, and the decrease was greater as the dose increased (Fig. 2). The ED_{50} was 330±27 ng (107.2±8.9 pmol)/100 g (n=23) (Fig. 4). The significant decrease was obtained at 100 ng/100 g. Compared to the vasodepressor effect, the bradycardia occurred a little more slowly and recovered more quickly, and the extent of the decrease was smaller. In 5 of 28 birds, injection of α-hANP caused tachycardia

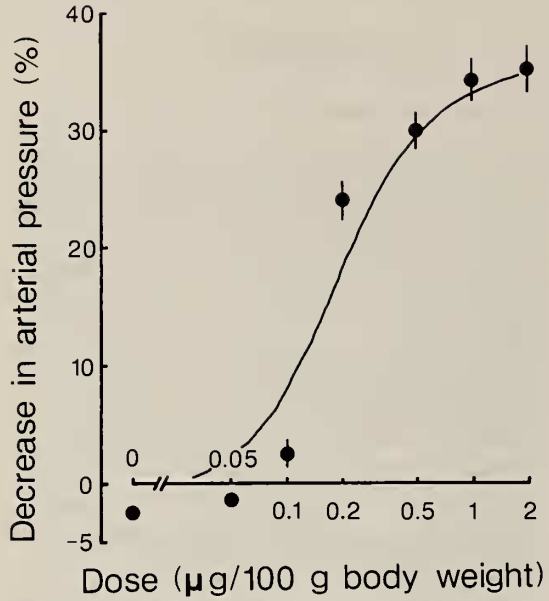

FIG. 3. Dose-response relationship for the vasodepressor effect of α-hANP in 28 quail. Doses are expressed in a log scale. The logistic curve that fitted best is shown in the figure. The calculated ED_{50} in 179±14 ng (58.2±4.6 pmol)/100 g body weight. The vertical bars represent standard errors of the mean.

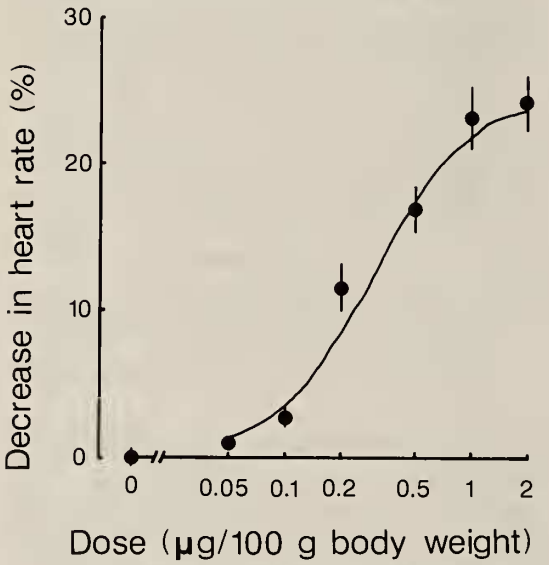

FIG. 4. Dose-response relationship for the bradycardial effect of α-hANP in 23 quail. Doses are expressed in a log scale. Among 28 quail examined, 5 quail showed tachycardia after injection of hANP. The logistic curve that fitted best is shown in the figure. The calculated ED_{50} is 330±27 ng (107.2±8.9 pmol)/100 g body weight. The vertical bars represent standard errors of the mean.

although hypotension was induced in the same birds. The basal heart rate of these birds (370±45 beats/min, n=5) was smaller than that of the other birds in which bradycardia was induced (561±19 beats/min, n=23).

Structure-activity relationships for analogs of hANP

As shown in Figure 5, sensitivity to hANP was well conserved and little tachyphylaxis was observed even after repetitive injections of α-hANP and its analogs. The hANP analogs with amino acids removed from the NH_2-terminus, hANP-(4-28), hANP-(5-28) and hANP-(7-28) were almost as potent as α-hANP in terms of their vasodepressor activity (Table 1). However, the peptides with amino acids removed from the COOH-terminus, hANP-(5-25) and hANP-(7-23), were less potent. No significant change in the vasodepressor potency was observed after the disulfide bond of hANP-(7-23)([$Cys^{7,23}$] hANP-(7-23)) was replaced by the ethylene linkage ([$Asu^{7,23}$] hANP-(7-23)). The modification of amino acids within the ring structure had variable effects (Table 1). When Met at the 12th position was replaced by Ile (rat ANP) or Nle, or when Gly at the 9th position was replaced by D-Ala, the potency did not change from that of the parent molecule. However, replacement of Asp at the 13th position with Asn, oxidation of Met at the 12th position, or removal of Gly at the 9th position, greatly decreased the potency of the peptide.

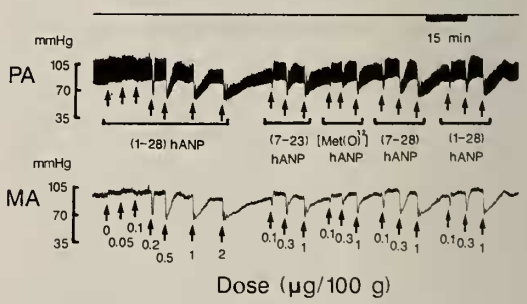

FIG. 5. Changes in arterial pressure (PA) and its mean (MA) after injection of hANP-(1-28) (α-hANP), hANP-(7-23), [Met(O)^{12}] hANP-(1-28), and hANP-(7-28) into a quail. The injection of α-hANP was repeated at the end of the experiment to confirm the reproducibility of data.

TABLE 1. Relative vasodepressor potencies of hANP analogs in the quail (n=6). Relative potencies by the spasomlytic activity in the chick rectum and rat aorta *in vitro* and by natriuretic activity in the rat *in vivo* are given for comparison. Values are means±SE of the mean. Amino acid sequence of hANP-(1–28) is:

1 5 10 15 20 25
S–L–R–R–S–S–C–F–G–G–R–M–D–R–I–G–A–Q–S–G–L–G–C–N–S–F–R–Y

Analogs of hANP	Potencies (%)			
	quail depressor	chick* rectum	rat* aorta	rat* natriuresis
(1-28)				
hANP	100	100	100	100
[Ile^{12}]	83.2± 9.7	122	226	236
[$Met(O)^{12}$]	15.8± 4.0	2	4	13
(4-28)				
hANP	86.3±10.7	64	79	97
(5-28)				
hANP	77.9±15.1	67	96	73
(7-28)				
hANP	123.5±16.2	364	154	148
[Nle^{12}]	82.5±15.3	169	61	141
[D-Ala^{9}]	119.0±15.6	356	120	107
[Asn^{13}]	18.8± 5.9	5	1	0
des-Gly^{9}	26.5± 6.9	1	0	0
(5-25)				
hANP	63.7±14.7	31	5	82
(7-23)				
hANP	50.7±12.7	139	3	27
[$Asu^{7,23}$]	60.3± 7.7	77	1	6

*[14]

DISCUSSION

A moderate dose of α-hANP caused a profound hypotension in the quail, as has been reported to occur in the chicken after injection of α-rat ANP [9]. In the chicken, a crude heart extract also decreases arterial pressure. Thus, ANP-like material appears to be present in the chicken heart, as suggested by the results of immunohistochemical analysis [20]. The presence of ANP-like material was also reported in the quail by immunohistochemistry using antibodies to pig ANP [21]. We also observed in preliminary experiments that the quail heart contained substances that cross-react with antibodies raised against α-hANP, and that the substance extracted had a potent vasodepressor effect in the quail (Takei and Ando, unpubl. data). However, the amino acid sequence of this immunoreactive ANP may differ from that of α-hANP, since the concentration in the heart extract determined by the quail vasodepressor bioassay was more than 10,000 times greater than the value measured by radioimmunoassay for hANP. Thus, hANP has a potent vasodepressor effect in the quail, although its molecule may be structurally different from quail ANP. Very recently, amino acid sequence of chicken ANP has been determined, whose sequence has only 52% homology to hANP [22].

In mammals, a bolus injection of α-hANP has

been shown to decrease arterial pressure in a linear fashion at doses between 33 and 333 pmol (0.1 and 1 μg)/kg in anesthetized dogs [23], and at doses between 0.18 and 9 nmol/kg in conscious, spontaneously hypertensive rats [24]. Values of ED_{50} were not calculated but dogs appear to be a little more sensitive to the vasodepressor effect of human (also native to dogs) ANP than are the rat and the quail. However, the maximal hypotension in birds (50%) was greater than that of mammals (10–20%).

It is known that, in mammals, an acute hypotension is useally followed by reflex tachycardia, which is mediated via sino-aortic baroreceptors [25]. Since profound hypotension was induced in all quail in the present study but bradycardia was induced in most birds after injection of hANP, hANP may have some inhibitory effect on heart rate. In guinea pigs. ANP also induces hypotension and bradycardia, but the effect of ANP on heart rate does not appear to be a direct action, since rat ANP-(5-28) did not change the frequency of spontaneously beating atrium *in vitro* [26].

For the expression of vasodepressor activity in the quail, amino acids at the COOH-terminus appear to be more important than those at the NH_2-terminus, and the modification of some amino acids within the ring structure, which may change the tertiary structure of the molecule, affects the potency. These results are similar to those obtained in the spasmolytic activity of the chick rectum and rat aorta, and in the natriuretic activity of rats (Table 1). However, some differences are observed in different preparations; in the chick rectum, removal of 6 amino acids from the NH_2-terminus increases the activity more than three folds, and in the rat preparations, the homologous rat peptide has more than two-fold activities than does the human peptide. Other major differences of the quail preparation from other preparations are that modifications of some amino acids within the ring structure cause smaller loss of activity, and that substitution of a disulfide bond with an ethylene linkage does not decrease the activity. Garcia *et al.* [16] showed that amino acids at the NH_2-terminus as well as those at the COOH-terminus are important for the spasmolytic activity in the rabbit aorta. For the antidipsogenic activity in the rat, the removal of amino acids from the NH_2-terminus does not change the activity, but the (7-23) peptide has no activity [15]. Collectively, it appears that quail vascular receptors can respond to a greater variety of ANP analogs than do other ANP receptors. Supporting this idea are our preliminary data which show that ANP-like substance extracted from carp hearts causes hypotension in the quail, but has no relaxant effect on the chick rectum (Uemura and Takei, unpubl. data). It appears that the quail vasodepressor effect is a usefil assay system for nonmammalian ANPs whose molecular structures may be different from mammalian ANPs.

In summary, the quail exhibited high sensitivity to the vasodepressor effect of hANP, and because of their small size (100 g), they can respond to as little as 50 ng (16 pmol)/bird. The vasodepressor effect was profound (50%), and reproducible even after repeated injections (see Fig. 5). Thus, it seems that the quail vasodepressor bioassay is considered to be a useful *in vivo* assay system for the comparative study of ANP in nonmammalian vertebrates.

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